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Mitochondrial Delivery Is Essential for Synaptic Potentiation

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Abstract. Mitochondria, as portable generators that power synaptic function, regulate the ATP supply and calcium homeostasis in the neuron. As molecular interactions within the synapses before and after the potentiation are beginning to be elucidated, the deciding moment during the tetanic stimulation that gives rise to the strengthening of the synapse remains a mystery. Here, I recorded electrically from an intact *Drosophila* nervous system, while simultaneously using time-lapse confocal microscopy to visualize mitochondria labeled with green fluorescent protein. I show that tetanic stimulation triggers a fast delivery of mitochondria to the synapse, which facilitates synaptic potentiation. Rotenone, an inhibitor of mitochondrial electron transport chain complex I, suppresses mitochondrial transport and abolishes the potentiation of the synapse. Expression of neurofibromin, which improves mitochondrial ATP synthesis in the neuron, enhances the movements of mitochondria to the synapse and promotes post-tetanic potentiation. These findings provide unprecedented evidence that the mitochondrial delivery to the synapse is critical for cellular learning.

Introduction

Mitochondria play a central role in a variety of cellular processes, including ATP production by oxidative phosphorylation, robust regulation of calcium homeostasis, generation of reactive oxygen species, and initiation of apoptosis (Morris and Hollenbeck, 1993; Wallace, 1999). The neuronal mitochondria vary constantly in their morphology and movement in response to changes in the local energy

state (Nicholls and Budd, 2000; Li *et al.*, 2004; Lisman and Spruston, 2005). Because the soma of the neuron is distant from its terminals, mitochondrial trafficking will substantially shape neuronal function (Chen and Chan, 2006).

The synchronization between mitochondrial conductivity and calcium dynamics in the presynaptic terminal suggests an active role of mitochondria in synaptic plasticity (Jonas *et al.*, 1999). The studies on Milton protein, dynamin-related protein (drp1), and mitochondrial GTPase dMiro suggest that unimpaired transport and distribution of mitochondria are required for normal synaptic transmission (Stowers *et al.*, 2002; Verstreken *et al.*, 2005; Guo *et al.*, 2005). In the case of drp1, the dependence of reserve-pool vesicular recruitment and mobilization on mitochondria indicates a specific role for mitochondria in regulating synaptic strength (Verstreken *et al.*, 2005). Finally, porin—a building block of the mitochondrial permeability transition pore—functions in both fear conditioning and spatial learning in mice (Weeber *et al.*, 2002) and in the selective up-regulation of mitochondria-encoded genes resulted from learning and memory (Pinter *et al.*, 2005). These findings suggest that changes in mitochondrial function could modulate behavioral plasticity.

Mitochondrial trafficking depends primarily on kinesins for anterograde transport and dyneins for retrograde transport (Goldstein and Yang, 2000). The rapid movements of mitochondria are microtubule-based and the slower movements are actin-based. It is reasonable to envision that mitochondria take an “express train” microtubule to the targeted synapse and employ “local taxi” actin for flexibility in reaching a precise location among the synaptic terminals (Hollenbeck, 1996).

The neuromuscular junction (NMJ) of *Drosophila* larvae offers an ideal model for studying the role of mitochondrial movements in synaptic plasticity in an intact nervous sys-

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Abbreviations: drp1, dynamin-related protein; EJP, evoked junction potential; GFP, green fluorescent protein; NF1, Neurofibromatosis-1 gene; NMDA, *N*-methyl-D-aspartate; NMJ, neuromuscular junction, PKA, protein kinase A; TMRE, tetramethylrhodamine ethyl ester.

tem because of its organized layout of the motor axons, its transparent muscle fibers, and its conservation of many key synaptic molecules that are also present in mammalian systems (Zhong, 1995; Saitoe and Tully, 2000). As in mammalian central synapses, glutamate is the primary excitatory neurotransmitter at the larval NMJ, with both presynaptic and postsynaptic biochemical signaling contributing to the modulation of synaptic strength. Several forms of synaptic plasticity have been described, including paired-pulse facilitation, short-term facilitation, augmentation, and post-tetanic potentiation—an enhancement of synaptic transmission lasting many minutes after an extensive high-frequency or tetanic presynaptic stimulation (Zucker, 1989). As with memory formation in adult flies, these forms of synaptic plasticity have been dissected by genetic and pharmacological manipulation at the larval NMJ (Zhong, 1995; Saitoe and Tully, 2000; Guo and Zhong, 2006). The NMJ preparation is, therefore, a genetically manipulable and optically accessible system that is useful for studying the role of mitochondria trafficking in synaptic potentiation.

My investigation started by categorizing different populations of mitochondria on the basis of their pattern of movements; recording the effects of motor nerve stimulation on mitochondrial movement and synaptic potentiation, combining time-lapse confocal microscopy with electrophysiological methods; and examining the correlation between the modulation of mitochondrial movements and synaptic potentiation, using rotenone and neurofibromin over-expression.

Materials and Methods

Drosophila stocks

Two mitochondrial GFP (*mito-GFP*) lines were used. The Pilling and Saxton *mito-GFP* line *W; D42-GAL4, p{w⁺mc}=UAS-mitoGFP* was generously given by Dr. Pilling and Dr. Saxton at Indiana University (Horiuchi *et al.*, 2005). This line carries a fusion gene with a *GAL4*-responsive promoter and a mitochondrial import sequence fused to GFP, and also a *D42-GAL4* promoter that produces motor-neuron-specific expression of this mitochondrial marker. The Cox *mito-GFP* line, which is constructed by fusing the human COX VIII mitochondrial targeting signal to the N terminus of enhanced green fluorescent protein (Cox and Spradling, 2003), was generously given to me by Dr. Rachel Cox from the Carnegie Institution of Washington. In the offspring from the cross between the Cox *mito-GFP* line and the *ELAV-GAL4* line (Bloomington Stock center), the neuronal mitochondria in the larva are labeled. Most of the data in this study were collected with the Pilling and Saxton *mito-GFP* line, which produces a stronger signal.

The *hsNF1/+* line (also called *hsNF1/mito-GFP*) was obtained from the cross between *hsNF1/hsNF1/+* flies and the Pilling and Saxton *mito-GFP* line. The motor neurons of

the *hsNF1/mito-GFP* flies express mito-GFP and neurofibromin by the leakage expression of a heat-shock-controlled *Neurofibromatosis-1* (*NF1*) transgene (Guo *et al.*, 2000; Tong *et al.*, 2007).

The *CD8-GFP* line was used to visualize the branching of the synaptic terminals at the neuromuscular junction (NMJ). The line was a gift from Dr. Mani Ramaswami at the University of Arizona, Tucson.

Neuromuscular junction preparation

Drosophila 3rd instar larvae were mounted in a recording chamber under Schneider solution (Sigma) perfusion at 25°C to be dissected dorsally to expose the ventral nerve cord. Data were collected from synaptic terminals that innervate muscle fibers 6, 7, 12, and 13. To co-stain synaptic mitochondria, the preparations were incubated for 10 min at 25°C with tetramethylrhodamine ethyl ester (TMRE, 10 nmol l⁻¹), a membrane-sensitive mitochondrial dye.

Image acquisition and analysis

Images were acquired by a Zeiss upright LSM510 META confocal microscope with both 40× (NA 1.2) and 63× (NA 1.4) water immersion objectives. For time-lapse imaging, confocal images of NMJ were collected at 0.5-μm steps throughout the entire *z* dimension, which takes about 1 min for the collection of the entire stack. The 40× lens was used in most experiments. Confocal images were then collapsed in the *z*-axis to yield two-dimensional projections. The movements of mitochondria were traced and measured manually at individual NMJ by MetaMorph software, ver. 7 (Universal Imaging). The recruitment of mitochondria to the terminals under tetanic stimulation was analyzed by a multi-object tracking paradigm customized in Metamorph software with manual confirmation. To analyze the redistribution of mitochondria at the synapse, mitochondrial density was recorded by measuring the number of mitochondria per micrometer at given time points during the recording. Statistical analysis was performed with Graphpad Prism software, ver. 4, and the Microsoft Excel statistical analysis.

Electrophysiology

The nerve terminals were stimulated by electric pulses (1 ms, 2 times threshold voltage) delivered to the appropriate segmental nerve at 10 Hz via a glass suction electrode (20-μm inside diameter). Synaptic potentiation was recorded and measured using a protocol adapted from Kuromi and Kidokoro (2000). In rotenone inhibition experiments, rotenone (1 μmol l⁻¹) was applied through the suction electrode that bathed the motor axon. Electrophysiological recordings were made along with the image acquisition.

Mitochondrial respiration and ATP synthesis rate

Mitochondria were isolated from adult flies of two lines: *hsNF1/+* (experimental) and *mito-GFP* (controls). In each case, 40 to 80 flies were gently crushed in a 10-ml Kontes homogenizer with seven strokes of the pestle in 3 ml of homogenization buffer consisting of 225 mmol l⁻¹ mannitol, 75 mmol l⁻¹ sucrose, 10 mmol l⁻¹ 3-(*N*-morpholino) propane sulfonic acid, 1 mmol l⁻¹ ethylene glycol tetraacetic acid, and 0.5% bovine serum albumin, pH 7.2, at 4°C. The extracts were filtered through eight layers of cheesecloth, and then centrifuged in a Beckman Avantis-J25 at 300 × *g* for 3 min. The supernatant was centrifuged at 6000 × *g* for 10 min to obtain a mitochondrial pellet. Mitochondrial protein was determined by the Bradford method, using Bio-Rad reagents; the values were corrected for the bovine serum albumin in the homogenization buffer. Respiration rates were determined by oxygen consumption using a Clark-type electrode and metabolic chamber containing 650 μl of reaction buffer consisting of 225 mmol l⁻¹ mannitol, 75 mmol l⁻¹ sucrose, 10 mmol l⁻¹ KCl, 10 mmol l⁻¹ Tris-HCl, and 5 mmol l⁻¹ KH₂PO₄, pH 7.2, at 25°C. Rates of ATP synthesis were calculated on the basis of ADP consumption and amount of oxygen consumption during mitochondrial respiration (Tong *et al.*, 2007).

Rotenone (1 mmol l⁻¹) was added to the isolated mitochondria in the metabolic chamber to measure the inhibition of ATP synthesis to be compared with untreated mitochondria as controls.

Results

Microanatomy of the motor axons, their synapses, and mitochondria

Mitochondria were genetically labeled with *mito-GFP* expressed in motor neurons of *Drosophila* larvae with motor-neuron-specific *D42-Gal4* driver (methods). The three-dimensional stacked, time-lapse confocal microscopy resolves individual mitochondria (longitudinal axis: 0.3–1.2 μm) and tracks their movements at different optical sections. TMRE, a membrane-sensitive mitochondrial dye, was used to co-stain synaptic mitochondria (Fig. 1a–c).

Categories of mitochondrial populations

Synaptic mitochondria can be categorized into two populations on the basis of their distinctive movement patterns. One is the “resident pool,” which consists of mitochondria that reside at the synaptic terminal and show limited movements. The other population is the “activated pool,” which consists of mitochondria that move to or from the terminal during the recording (see Movie 1 at <http://www.biolbull.org/supplemental/>). The percentage of activated mitochondria within the total terminal population was 21.4% ± SD 2.3% (*n* = 17 terminals from 9 larvae). Among the activated

mitochondria, 51.2% ± 2.6% showed anterograde movement toward the synaptic terminal, and 49.4% ± 3.1% showed retrograde movement away from the terminal (*P* = 0.14 comparing anterograde and retrograde mitochondria).

Motor nerve stimulation increases mitochondrial density at the synapse in potentiation

I used a combination of time-lapse confocal mitochondrial tracking and dual sharp-electrode voltage-clamp technique to record evoked junction potential (EJP) at the NMJ (Fig. 1d). Tetanic stimulation (10 Hz) of the motor nerve increased the delivery of mitochondria to the synapse (Fig. 2a; Movie 2 at <http://www.biolbull.org/supplemental/>). The immediate bifurcation point (20–30 μm upstream from the synapse) was chosen as the starting point for recording the movements of mitochondria. In response to tetanic stimulation, mitochondrial density was significantly increased at the stimulated synapses while mitochondrial density in control synapses remained steady throughout the experiment. The potentiation of the synapse, demonstrated as an increase in EJP, followed the accumulation of mitochondria at the synapse (Fig. 2a).

Actions of rotenone and neurofibromin

Effects on the respiration of isolated mitochondria. Application of a potent complex I inhibitor, rotenone (1 μmol l⁻¹), in the suction electrode that bathed the projecting axon loop (Fig. 2) blocked both the delivery of mitochondria to the synapse and the potentiation of the synapse (Fig. 2). Neurofibromin expression by *hsNF1* transgene increased mitochondrial respiration by enhancing complex I activity (Tong *et al.*, 2007), which increases the mitochondrial ATP production rate by 54% (Fig. 3).

Effects of neurofibromin on mitochondrial movements and on synaptic potentiation. Neurofibromin expression did not alter the density of mitochondria in the NMJ synapses before tetanic stimulation of the preparation (Fig. 2a); however, in response to stimulation, neurofibromin expression in these neurons promoted a fast elevation of the synaptic mitochondrial density. The mitochondrial density in elevated neurofibromin terminals was significantly higher than that in the control synapses that are potentiated (Fig. 2a). EJP amplitudes in synapses with neurofibromin over-expression were also increased above those of potentiated control synapses (Fig. 2a). Moreover, after the initiation of tetanus stimulation, both mitochondrial accumulation and synaptic potentiation occurred 5 min earlier in synapses over-expressing neurofibromin than in control synapses (Fig. 2).

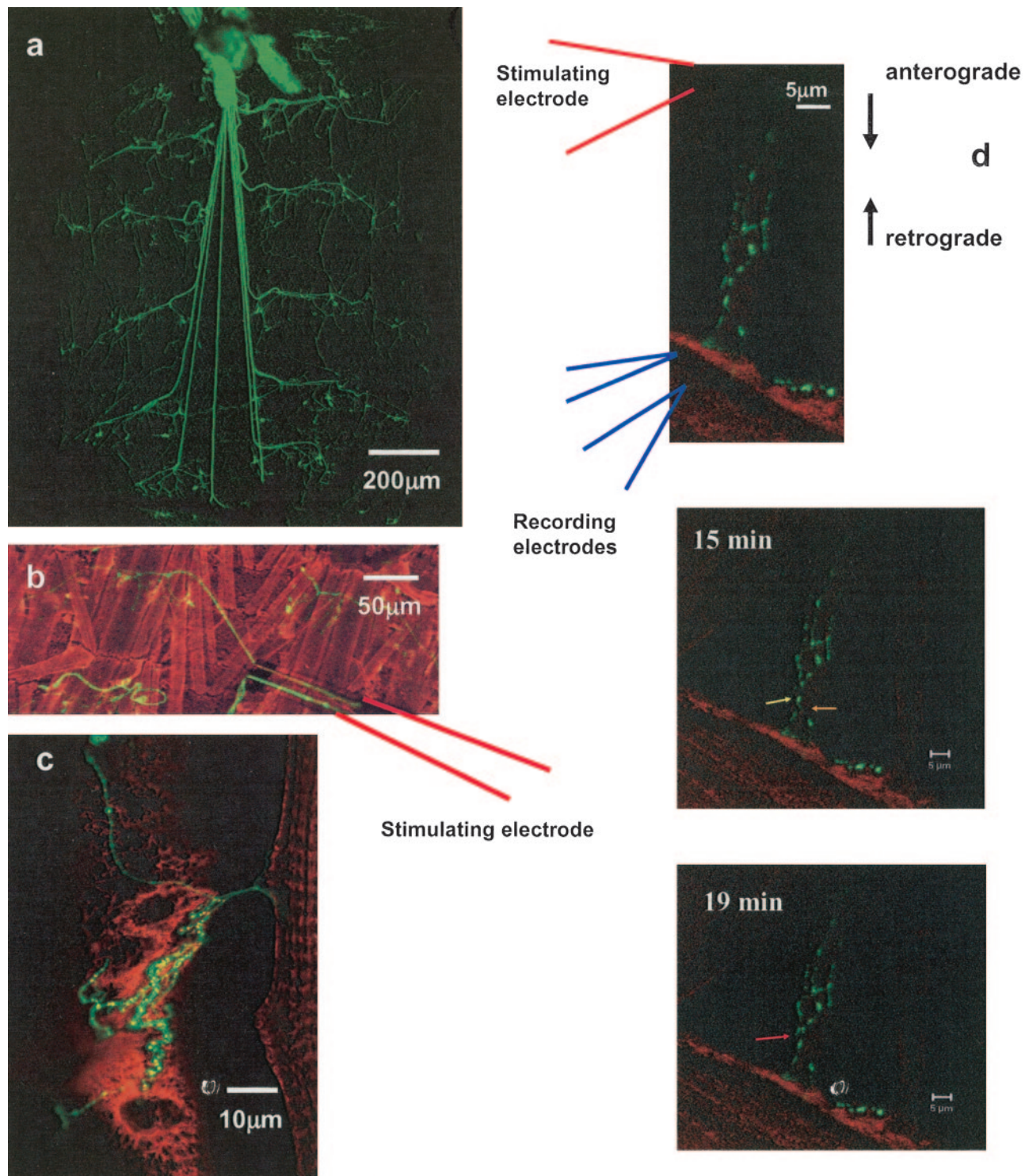


Figure 1. Microanatomy of motor axons, synaptic terminals, and mitochondrial movements under synaptic stimulation. (a) *Drosophila* larval motor nerves labeled by expression of motor-neuron-specific *mito-GFP*. (b) Suction electrode (red) is positioned to stimulate projecting axon loop of the motor nerve innervating the synaptic terminal. This suction electrode was also used to apply rotenone to the axons. Muscle fibers have a salmon color in the presence of TMRE. (c) Mitochondrial distribution (bright orange dots, TMRE labeled) in the synaptic terminals of *CD8 GFP* line flies (green) branching over muscle fibers at NMJ. (d) GFP-labeled neuronal mitochondria at the presynaptic terminal with schematic arrangement of stimulation and recording electrodes. The movements of individual mitochondria are indicated at 15 min and 19 min, the yellow and orange arrows identify two anterograde mitochondria, and the red arrow marks one retrograde mitochondria (see Movie 2 at <http://www.biolbull.org/supplemental/>).

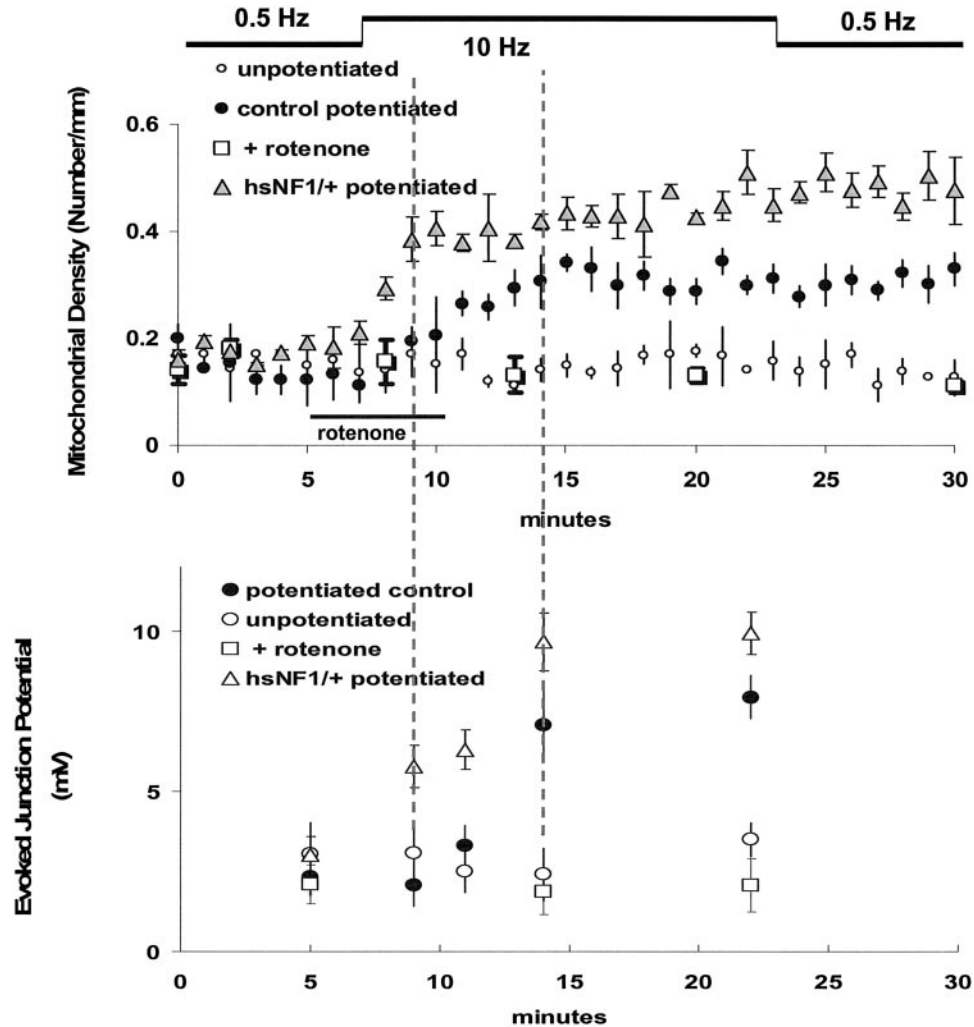


Figure 2. Correlation between mitochondrial movements and synaptic potentiation. Upper panel: Mitochondrial density (M.D.) is the number of mitochondria per micrometer measured because the diameter of nerve branches at the NMJ presynaptic terminal appears to be constant. The density is presented in terms of length rather than volume. M.D. of the control-potentiated terminal (●) is compared with the section-matching contralateral unpotentiated terminal (○). Density increase indicates a net increase of mitochondrial flow toward the synapse. Data points are means \pm SD ($n = 8$ terminals). There is no significant difference before 10 min ($P = 0.13\text{--}0.21$). Significant difference between unpotentiated and potentiated synapses is detected after 10 min ($P < 0.001$). Lower panel: Evoked synaptic potentials (EJPs) were measured and plotted. The M.D. and EJP measurements under rotenone ($1 \mu\text{mol l}^{-1}$) are shown (□) with the bar (—) indicating the application of rotenone *via* suction electrode from 5 min until 10 min for both panels. Significant differences were detected between control and potentiated EJP amplitude at 14 min ($P < 0.001$) and 22 min ($P < 0.001$). The dashed lines indicate the timing between potentiation occurrence and mitochondrial density increase, comparing control synapses to neurofibromin expression synapses. Neurofibromin enhanced the delivery of mitochondria to the synaptic terminals, demonstrated as a significant elevation of mitochondrial density during tetanic stimulation, correlated with an increased EJP in the *hsNF1/+* synapses (▲) at 9, 11, 14, and 22 min compared to both the unpotentiated ($P < 0.001$) and control potentiated synapses ($P < 0.01$ at 9 and 11 min, $P < 0.02$ at 14 and 22 min). Stimulation: 1 ms, $2\times$ threshold, 10 Hz; begins at 7 min, ends at 23 min. Data collected each minute.

Discussion

This study demonstrates, for the first time in an intact nervous system, that tetanic stimulation triggers the delivery of mitochondria to specifically targeted synapses, and that

the resulting reorganization of the mitochondrial network may be a prerequisite for synaptic potentiation. The analysis of mitochondrial movement indicates that the net increase in mitochondrial density seems to be mainly due to an increase in the rate of mitochondrial anterograde movement towards

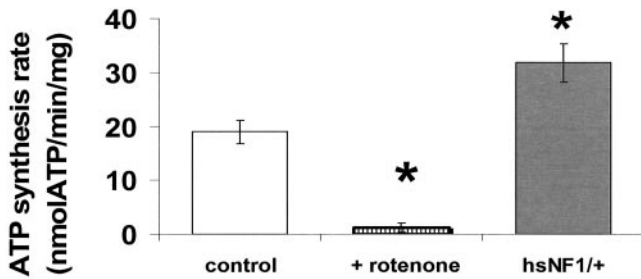


Figure 3. Mitochondria isolated from *hsNF1/+* flies have a higher rate of ATP production than control. Rotenone ($1 \mu\text{mol l}^{-1}$) added to *hsNF1/+* mitochondria reduced the rate of ATP production. Asterisk (*) indicates that rates are significantly different from control mitochondria isolated from *mito-GFP* lines, $P < 0.01$, $n = 5$.

the synapse. The unprecedented finding that the synapse is potentiated when mitochondrial dynamics are elevated by neurofibromin expression, which leads to an increased ATP production, suggests a mitochondrial approach to the enhancement of learning and memory.

The physiological significance of anterograde and retrograde mitochondria invites further scrutiny. Although the retrograde mitochondria may be recycled or degraded after their service at the synapse, the anterograde mitochondria may accommodate the energy demand of synaptic potentiation. These newly delivered mitochondria may remodel the calcium homeostasis and the energy capacity required to sustain the potentiated synaptic activity, with possible preparation for long-term modification of the synapse. Genetic marking of an individual anterograde or retrograde mitochondrion will provide further insight into the destination of these mitochondria and their active roles in shaping the function of their host neuron.

The proximate signal that triggers the increased rate of mitochondrial delivery to the activated synapses remains to be elucidated. In determining whether the insertion of mitochondria into the synapse is sufficient to initiate synaptic potentiation, the challenge will be to manipulate the mitochondria into a silent synapse without using electric stimulation and then test whether synaptic strengthening occurs.

There is tremendous potential to employ these real-time *in vivo* imaging analyses that investigate the synchronicity of neuronal and behavioral dynamics at both the cellular and behavioral levels as a means to characterize phenotypes of biomedical interests (Bokil *et al.*, 2006; Misgeld and Kerscheneister, 2006).

The *Neurofibromatosis-1* gene (*NF1*) regulates the adenylyl cyclase and protein kinase A (AC/PKA) pathway and Ras signaling (Guo *et al.*, 2000; Tong *et al.*, 2002). The over-expression of neurofibromin increases ATP production by elevating complex I activity through PKA phosphorylation of complex I subunits (Papa, 2002; Tong *et al.*, 2007). Flies with neurofibromin over-expression also exhibit prolonged olfactory memory retention (unpubl. data). Further

experiments are warranted to investigate whether mitochondrial dynamics are active in several learning and memory-enhancement manipulations such as the expression of cAMP-responsive element-binding protein (CREB) in *Drosophila* (Yin *et al.*, 1995) and the over-expression of adenylyl cyclase (Wang *et al.*, 2004) and NMDA receptor 2B (Tang *et al.*, 1999) in mice.

Mitochondrial dynamics, in a more general sense, encompasses mitochondrial functional fluctuation, movements, fusion and fission of individual mitochondria, and reorganization of the mitochondrial network. If the unique temporal and spatial dynamic patterns of mitochondrial distribution are a “mitochondrial memory code” that dictates the potentiation of specific synapses, the plasticity of the neuronal network, and ultimately a decisive modification of behavior, the disruption of the “code”—manifested as functional aberration and movement disruption—will inevitably result in an energy imbalance, loss of homeostasis, and deficiency of synaptic plasticity that underlie the cognitive deterioration seen in pathological conditions and aging.

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